Original Article

Prophylaxis against renal ischemia-reperfusion injury in canine model: Stem cell approach

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ABSTRACT

Introduction: Stem cell therapy at the time of ischemia/reperfusion (I/R) injury has been hypothesized to attenuate the severity of acute kidney injury and to accelerate the regeneration process in lower animal models. Data in higher animal models is limited and discordant. We aimed to explore the reno-protective effects of stem cells on I/R related renal injury in a canine model.

Materials and Methods: Twenty-seven dogs that were treated with bone marrow-derived mesenchymal stem cells (BM-MSCs) were compared with another 27 dogs treated with adipose tissue-derived MSCs (AT-MSCs) following 90 min of warm ischemia to assess IR injury. Each group was divided into three subgroups (nine dogs each), according to the stem cell dose (5, 10, 15 × 10^6 in 500 µl volume) injected directly into the renal cortex after reperfusion. All dogs were re-evaluated by renogram, histopathology, and pro-inflammatory markers at 2 weeks, 2, and 3 months.

Results: In Group I, there was a mean reduction of creatinine clearance by 78%, 64%, and 74% at the three used doses, respectively, at 2 weeks. At 3 months, these kidneys regained a mean of 84%, 92%, and 72%, respectively, of its basal function. In Group II, the reduction of clearance was much more modest with mean of 14%, 6%, and 24% respectively at 2 weeks with more intense recovery of renal function by mean of 90%, 100%, and 76%, respectively, at 3 months. Group I had significantly more tubular necrosis and delayed regeneration compared with the Group II. Expressions of pro-inflammatory markers were upregulated in both the groups with a higher and more sustained expression in Group I.

Conclusion: Stem cells protected against ischemic reperfusion injury in a canine model. AT-MSCs provided better protection than BM-MSCs.

INTRODUCTION

Acute kidney injury (AKI) represents a frequently confronted clinical scenario with significant subsequent morbidity and mortality. AKI management comprises of a series of supportive measures without a specific insult-oriented therapy. Ischemia/reperfusion (I/R) events are a frequent cause of AKI resulting in immune and metabolic consequences in renal tissues.[1] In routine urologic practice, renal ischemia with subsequent I/R is deliberately induced during kidney transplantation, partial nephrectomy, and anatrophic nephrolithotomy with subsequent early renal dysfunction.

In the last decade, urologic literature is replete with data supporting the use of mesenchymal stromal cells (MSCs) in cases of I/R insult. The administration of MSCs has been hypothesized to decrease AKI severity and to hasten the regenerative process in lower animal models.[2,3] Limited and controversial data are also available in the higher animal models.[4-6] The role of bone marrow-derived mesenchymal stem cells (BM-MSCs) compared with adipose tissue-derived

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MSCs (AT-MSCs) in renal function restoration after renal ischemic insult is still undefined.[7-9] Finally, the dose adjustment of injected cells is poorly studied.[10] In this study, we aimed to critically analyze the protective effects of stem cells on minimizing I/R injury in a higher animal canine model.

MATERIALS AND METHODS

After obtaining the ethics committee approval for the study (STDF 4713-2014-154), dogs were purchased from the veterinary department of the university and maintained in quarantine for cleaning, feeding, and preparing for the operation for at least 2 weeks. A group of 60 dogs were utilized to perform kidney injury model. Their weights were in the range of 15–18 kg to be eligible for the surgery. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The animals had normal renal function before the study as confirmed by diuretic renogram.

Autologous canine MSCs were isolated with gentle pipetting which resulted in the generation of a single cell suspension. Stem cells were then counted and plated in a concentration of 10×10^6/ml in T-75 flasks. The cells were then cultured in a medium containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere that contained 5% CO_2. Medium was changed after 4 days and then every 3 days thereafter. Nonadherent hematopoietic cells were removed when the medium was changed. After a mean of 7 days, cells reached subconfluence and were detached with trypsin/ethylenediaminetetraacetic acid, were reseeded at 4×10^3 cells/cm^2, and were used for experiments after the third passage. MSCs features were demonstrated by typical spindle-shaped morphology and phenotypic characterization. The counted number of stem cells were inoculated with BrdU and injected directly into the renal cortex.

General anesthesia was induced by intravenous (IV) injection of 30 mg/kg dose of phenobarbital sodium. Animals were given 600 ml 0.9% saline IV during the surgical procedure. Warm ischemia (WI) was performed by open occlusion of the left renal artery with a vascular clamp to obtain complete ischemia. Ischemia was maintained for 90 min in 57 dogs and no reno-protective agents were administered to prevent/reduce the ischemic insult. The canine models were divided into 2 groups, Group I included 27 dogs that were treated with BM-MSCs whereas Group II (27 dogs) were treated with AT-MSCs. Each group was divided into three subgroups (9 dogs each), according to the stem cell dose (5, 10, 15×10^6 in 500 μl volume) injected directly into the renal cortex after reperfusion. Three dogs were positive control without MSC treatment, while three other were sham operated. In each subgroup, dogs were sacrificed at 2 weeks, 2, and 3 months (three dogs at each time interval). All dogs were evaluated by renogram prior to sacrifice to evaluate the percentage reduction in left renal function as compared to the baseline. Technetium 99 m-mercaptoacetyltriglycine was used for imaging as it is suitable for patients with renal insufficiency (subjects of this study).

Kidneys were harvested and pathologically evaluated. Routine staining with hematoxylin and eosin of 4 μm sections was performed. The light microscopic examination was reviewed by a pathologist uninformed about the experimental details. For light microscopic examination, the pathologist divided the kidney into two main parts – cortex and medulla. Further, subdivisions were made for medulla into outer strip outer medulla (OSOM), inner strip outer medulla (ISOM), and inner medulla. Inside each subpart, glomeruli were observed for hypercellularity. Tubules were observed for necrosis, atrophy, and regeneration. Regeneration indicators were: mitosis, solid tubules, solid sheets, and irregular dilated tubule. For the interstitial tissues, fibrosis and cellular infiltrate were identified. Tumor necrosis factor (TNF) and CD95 were localized in injured tissue by immunohistochemical staining based on the avidin-biotin-peroxidase method, as the apoptosis markers while collagen Type III was detected as a marker for tubulointerstitial injury.

The total RNA was isolated from the renal tissues using TRIzol reagents for the purpose of gene expression in the injured and the treated renal tissues. The expression of hypoxia-inducible factor, angiotensin II (ANG II) and TNF-α in the injured renal tissues was evaluated using RT^2-polymerase chain reaction. All measurements were performed in triplicates, and values are presented as means ± standard deviation.

RESULTS

In Group I, there was a reduction in creatinine clearance of the investigated kidney by a mean of 78%, 64%, and 74% for the three used doses respectively at 2 weeks. At 3 months, these kidneys regained a mean of 84%, 92%, and 72% respectively of their basal function. In Group II, the reduction of clearance was much more modest with a mean of 14%, 6%, and 24% respectively at 2 weeks with more intense recovery of the renal function at 3 months by mean of 90%, 100%, and 76%, respectively. Positive control showed more intense reduction of clearance by 90% at 2 weeks and regained 70% of the basal function at 3 months. Gradual improvement in clearance with time was observed even in the untreated control group. Nevertheless, treatment with stem cells resulted in lesser renal injury with faster recovery. The impact was more pronounced with AT-MSCs compared with BM-MSCs and the best outcome was achieved with the concentration of 10×10^6. Table 1 shows the mean percentage reduction in the clearance

Table 1: The mean percentage reduction in clearance in gamma camera measurement of the left kidney after 90 min ischemia after different time intervals

Cell type	Control (%)	MSC (5 M) (%)		MSC (10 M) (%)		MSC (15 M) (%)	
		BM	AT	BM	AT	BM	AT
2 weeks	90	78	14	64	6	74	24
2 months	32	56	11	26	6	47	22
3 months	30	16	10	8	0	28	24

M=Millions, MSC=Mesenchymal stem cells, BM=Bone marrow, AT=Adipose tissue

reduction on gamma camera measurement of the left kidney after 90 min ischemia after different time intervals in BM-MSCs and AT-MSCs treated groups, respectively.

On histopathological examination of the cortex in Group I, injected with BM-MSCs at different concentrations at different time intervals, apoptosis, dilated irregular tubules, loss of brush borders, and casts were seen maximum at 2 weeks and declined afterwards [Figure 1]. Similarly, mitotic figures appeared at 2 weeks and reached a maximum at 2 months. There were some regenerative changes in the form of prominent nucleoli with some solid tubules which were more prominent at 2 months. The regeneration was most prominent at the 5×10^6 and 10×10^6 concentrations. The tubular atrophy was noticed at all the time intervals but was more pronounced in the 2 weeks group. Interstitial inflammatory infiltrate was detected at all the time intervals with no interstitial fibrosis. Parallel changes were observed in Group II treated with AT-MSC and the best regenerative power was achieved with 10×10^6 concentration.

On examining, the OSOM in Group I, there were more apoptosis of tubules, loss of brush border, tubular necrosis with dilated irregular tubules and casts at 2 weeks as compared with that at the other time intervals. While tubular atrophy was noticed maximum in the 2-month model, interstitial infiltrate, and mild fibrosis was observed at 3 months. The regenerating tubules were more pronounced on the 3 months model in the form of solid sheets and tubules in addition to mitotic figures, with least regeneration at 2 weeks. The regeneration was more pronounced with the 5×10^6 and 10×10^6 dose concentrations compared with the 15×10^6 dose concentration. In Group II, regeneration started early at 2 weeks, and the response was more pronounced with 15×10^6 and 10×10^6 dose concentrations as compared to that the 5×10^6 dose concentration [Figure 2].

On examining, the ISOM in Group I, there were tubular apoptosis, dilated irregular tubules, loss of brush border, and casts involving the 2 weeks group, while only the tubular atrophy was seen in the 2 and 3 months, groups. Features suggestive of regeneration as mitotic figures, solid tubule, and dilated irregular tubules with prominent nucleoli started to appear at 2 months. The regeneration was maximal in the 10×10^6 group. The interstitial infiltrate

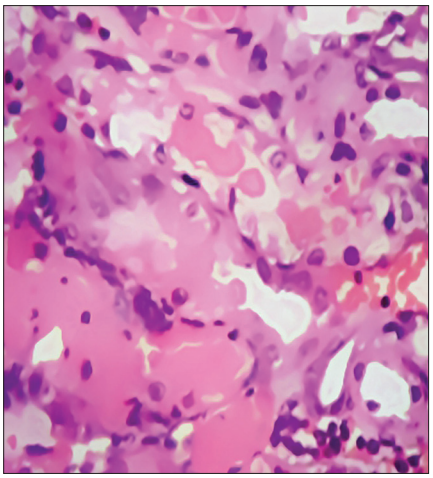

Figure 1: Significant tubular necrosis in superficial cortex in Group I (15 million subgroup) at 2 weeks; H and E, ×400

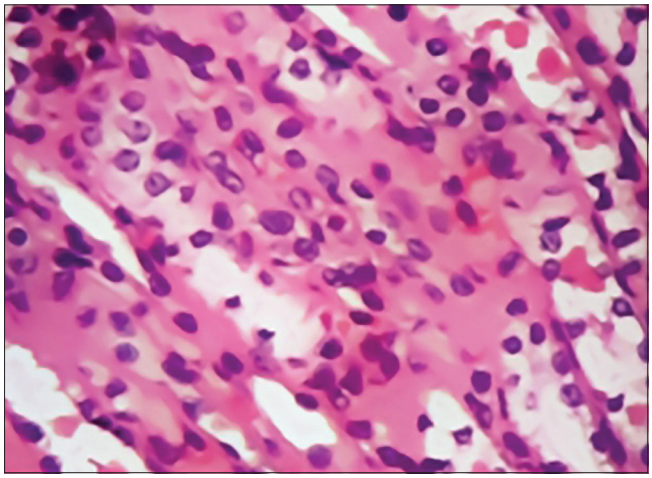

Figure 2: Regenerating solid sheets in outer medulla in Group II (10 million subgroup) at 2 months; H and E, ×100

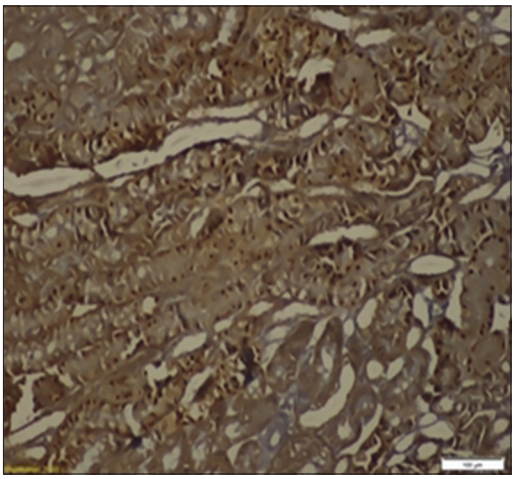

Figure 3: Severe inflammatory cells infiltrating the tubules with positivity to tumor necrosis factor in outer medulla in Group I (5 million subgroup) at 3 months. ×100

and fibrosis were noticed at all the time intervals [Figure 3]. Similar changes were reported in Group II with more abundant mitotic figures and regenerating solid sheets at

2 and 3 months. Equal regenerative power was observed between the 5×10^6 and 10×10^6 dose concentrations in this group.

On examining, the inner medulla in Group I, there was tubular degeneration at 2 weeks while tubular atrophy was noticed at the 2 and 3 months. The regenerative changes in the form of prominent nucleoli and mitotic figures were noticed in the 2 and 3 months groups and mostly with the dose of 10×10^6 cells. Interstitial inflammation was also noticed at all the time intervals, but interstitial fibrosis was observed only in the 3 months group. In Group II, regeneration started as early as 2 weeks with both 5×10^6 and 10×10^6 dose concentrations. The histopathologic insult was more apparent in the control group with a greater delay in recovery.

Our results revealed decrease in TNF-α expression, and this decrease strongly correlated with both the dose of stem cells injected and the type (either bone marrow or adipose tissue), as compared with the control group. Notably, this impact was apparent after 3 months in the bone marrow group, but it appeared at 1 month in adipose tissue group. Also, this impact appeared to be proportional with the dose of injection. Similarly, the data showed a decrease in ANG II expression with both types of stem cells utilized compared with the control group. Unlike the TNF, the reduction was more pronounced with the BM-MSCs group compared with the AT-MSCs group, especially with the 10×10^6 dose. Equal expression was observed utilizing the HIF in this experimentation that appeared to be proportional with the dose of injection. Notably, the sham-operated control group showed no clearance or histopathological changes whilst the pro-inflammatory markers were not examined in this group for cost-related issues.

DISCUSSION

Renal tissues have a remarkable ability to regenerate following injury, as it is not a terminally differentiated organ. However, this regenerative potential is related to the magnitude of insult and might be incomplete. If the insult is sustained, progressive and irreversible, fibrosis and scarring are inflected resulting in end-stage renal disease.[11] MSCs represent a heterogeneous population of adult multipotent cells and show a wide range of ability to differentiate into tissues of mesodermal lineages.[1] MSCs administration at the time of renal insult was proven to decrease AKI severity and to enhance kidney recoverability[2,3,5,6] based on their immunomodulatory, anti-inflammatory, and tissue repair properties. This effect can be attributed to communication mechanisms involving microvesicles. During MSCs therapy, the delivery of proteins, messenger RNA, and micro-RNA to tubular cells and may induce *de novo* expression of factors involved in cellular proliferation and repair, such as HGF.[12]

A large amount of evidence is available supporting the role of MSCs derived from bone marrow, adipose tissue, or even from skeletal muscles in enhancing renal protection against I/R injury in small animal models.[2,3] Stem cells were proven experimentally to prevent renal disease progression and even improve renal function in renovascular hypertension rat models.[13,14] In two different higher animal models, this effect was not sustained. Limited protective efficacy of MSCs was observed in a porcine model following AKI induction.[4] Similarly, MSCs did not exhibit reparative or paracrine protective properties in the sheep model.[15] While others showed evidence of decreased inflammation, apoptosis, and fibrosis with improved renal hemodynamics and function.[5,6] Our experiment is the first of its kind to be carried out in a canine model showing a significantly improved outcome with both types of MSCs utilized compared with the control group. Decades ago, the canine model was utilized to study the permissible warm renal ischemia time.[16] Canine nephron segments show remarkable similarity to nephrons of human kidney[17] Moreover, the canine model was suggested as a good model system for studying renal regeneration.[18] We chose a paired canine model with 90 min of WI to be a perfect example to study I/R injury as complete spontaneous recovery is not guaranteed.[19]

BM-MSCs are the most well-defined type of MSCs and have been tested in several studies for a wide range of therapeutic applications.[20] Nevertheless, the collection of bone marrow aspirate is a painful invasive procedure, can be accompanied by possibility of sepsis and sometimes yields limited number of MSCs .[21] On the other hand, MSCs isolated from adipose tissue have been suggested as an elegant cell source for regenerative medicine as adipose tissue is widely available for cell harvesting .[22] On exploration, the differences between both sources of MSCs, huge debate was observed. MSCs of adipose origin was reported to show better proliferation rates when extracted from pigs[7] while in humans, the MSCs of bone marrow origin showed better proliferation rates.[8] Regarding cell viability and growth factors' release, Juhl *et al*. showed that both BM-MSC and AT-MSC fulfilled the International Society for Cellular Therapy criteria after media expansion.[9] There is a significantly lower secretion of insulin-like growth factor with AT-MSCs compared with BM-MSCs under hypoxic conditions whereas the vascular endothelial growth factor/fibroblast growth factor secretions were found to be significantly higher with AT-MSCs.[23] Others showed that BM-MSCs are superior to adipose cell origin regarding their endothelial differentiation capabilities and paracrine action side by side *in vitro*.[24]

We showed the superiority of AT-MSCs over BM-MSCs in recovery of kidney function based on both the histopathological criteria as well as the recovery of renal function as estimated by renographic clearance. This is consistent with the report from Sullivan *et al*., who compared AT-MSCs with BM-MSCs when extracted from

a similar canine model similar to our study.[25] They showed that BM-MSCs yielded higher absolute cell numbers on average, while the adipose tissue yielded more consistent results. Interestingly, in the higher animal model, BM-MSCs failed to improve renal function following I/R insult.[4] On the other hand, others reported marvelous outcomes with AT-MSCs.[6]

Mode of stem cell administration may influence their localization into damaged tissues as MSCs are trapped in the tissue capillaries of lung, spleen, and liver.[26] Cellular injection through the distal thoracic aorta is beneficial compared with IV administration as it bypasses the pulmonary circulation. On the other hand, the intravascular administration of MSCs may lead to prothrombotic events.[27] Moreover, Burst *et al*. questioned the ability of MSCs injected in the renal artery in ameliorating renal damage in ischemic AKI.[28] For these reasons, different routes of MSCs administration have been verified in animal models of renal insult. Direct MSCs implantation into the renal parenchyma[29] and injection into the renal subcapsular region[30] has been proven to be efficient. We elected to inject the MSCs directly into the renal parenchyma immediately after release of the vascular clamp to have the highest concentration possible in the parenchyma. We identified only one study that evaluated the value of MSCs dose. Zhuo *et al*. found no difference between the 1×10^6, 2×10^6, and 5×10^6 doses in alleviating the ischemic insult in the rat model.[10] We elected to use higher doses in the canine model. Unlike the former experience, we observed dose based differences. We found that the 10×10^6 is the best therapeutic dose. It might be logical that increasing the dose may result in better outcomes, however, it was unclear why the 15×10^6 dose (highest) failed to produce further improvement. Limitation of the study included the small sample size that hindered a sound statistical analysis and impair robust interpretation of the data, particularly for stem cell dosage.

CONCLUSION

Stem cells can protect against I/R injury in a canine model. AT-MSCs provided better protection than BM-MSCs. The 10 million dose of MSCs is the best dose in ameliorating the ischemic injury.

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